FINAL REPORT

Study Title

In Vitro Mammalian Cell Micronucleus Assay in Human Peripheral Blood Lymphocytes (HPBL)

Testing Guidelines
OECD Guideline 487 (updated and adopted 26 September 2014)

Test Substance

JA900-DAA

Author

Shambhu Roy, Ph.D.

Study Completion Date
09 June 2015

Testing Facility

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AE19WX.348.BTL

Sponsor

International Flavors & Fragrances Inc.
800 Rose Lane
Union Beach, NJ 07735

TABLE OF CONTENTS

PAGE

STATEMENT OF COMPLIANCE

Study No. AE19WX.348.BTL was conducted in compliance with the US EPA GLP Standards 40 CFR 792 (TSCA) with the following exceptions:

1. The identity, strength, purity, stability and composition or other characteristics to define the test substance were determined by the Sponsor. However, the characterization documents do not indicate the regulations under which the analyses were conducted.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test substance as supplied.

2. Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

   Study Director Impact Statement: The impact cannot be determined because the appropriate analyses were not performed. The study conclusion was based on the nominal dose levels as documented in the study records.

Shambhu Roy, Ph.D. — Date
Study Director

QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AE19WX.348.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
07-Apr-2015	10-Apr-2015	Protocol Review	10-Apr-2015	10-Apr-2015
11-May-2015	11-May-2015	Evaluation of Cytotoxicity	11-May-2015	11-May-2015
26-May-2015	28-May-2015	Data/Draft Report	28-May-2015	28-May-2015
08-Jun-2015	08-Jun-2015	Final Report	08-Jun-2015	08-Jun-2015

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

For a multisite study, test site QA Statements are located in the corresponding contributing scientist report.

E-signature

Quality Assurance: Ellen Burns 08-Jun-2015 9:24 pm GMT

Reason for signature: QA Approval

Printed by:Ellen Burns

Printed on:8-Jun-15

STUDY INFORMATION

Study Conduct

Sponsor:	International Flavors & Fragrances Inc. 800 Rose Lane Union Beach, NJ 07735
Sponsor's Authorized Representative:	Xiao Huang, Ph.D.
Testing Facility:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study No.:	AE19WX.348.BTL

Test Substance

Identification:	JA900-DAA (The test substance is a polymer, 51% in ethanol.)
Synonym:	Jeffamine ED900 diacrylamide
Lot No.:	RDLV28986
Purity:	51.4% (per Certificate of Analysis)
Average Molecular Weight (M_n):	Approximately 1000 g/mol (provided by the Sponsor in the protocol) (Actual M_n = 1082, per Certificate of Analysis)
Description:	Clear colorless liquid
Storage Conditions:	Room temperature, protected from light
Receipt Date:	24 March 2015

Study Dates

Study Initiation Date:	07 April 2015
Experimental Start Date:	09 April 2015
Experimental Completion Date:	15 May 2015

Key Personnel

Study Director:	Shambhu Roy, Ph.D.
Testing Facility Management:	Rohan Kulkarni, MSc, Ph.D. Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Vanessa Johnson-Peltier, B.S.
Report Writer:	Meena Jois, B.S.

SUMMARY

The test substance, JA900-DAA, was tested in the *in vitro* mammalian cell micronucleus test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of an Aroclor-induced S9 activation system. A preliminary toxicity was performed to establish the dose range for testing in the micronucleus test. The micronucleus assay was used to evaluate the aneugenic and clastogenic potential of the test substance. In both phases, HPBL cells were treated for 4 and 24 hours in the non-activated test system and for 4 hours in the S9-activated test system. All cells were harvested 24 hours after treatment initiation. Dose formulations were adjusted to compensate for the purity (51.4%) of the test substance, using a correction factor of 1.95.

Water was used as the vehicle based on information provided by the Sponsor, the solubility of the test substance, and compatibility with the target cells. In a solubility test conducted at BioReliance, the test substance formed a clear solution in sterile water at a concentration of approximately 50 mg/mL, the maximum concentration tested for solubility. The formulation prepared for use in the solubility test also was adjusted for test substance purity using a correction factor of 1.95.

In the preliminary toxicity assay, the doses tested ranged from 0.2 to 2000 µg/mL. Cytotoxicity [55 ± 5% cytokinesis-blocked proliferation index (CBPI) relative to the vehicle control] was not observed at any dose level in any of the treatment groups. Based on these findings, the doses chosen for the micronucleus assay ranged from 250 to 2000 µg/mL for all three treatment groups.

In the micronucleus assay, cytotoxicity (55 ± 5% CBPI relative to the vehicle control) was not observed at any dose level in any of the treatment groups. The highest dose analyzed under each treatment condition was the highest dose tested in the definitive assay, which met the dose limit as recommended by testing guidelines for this assay. A minimum of 1000 binucleated cells from each culture were examined and scored for the presence of micronuclei.

The percentage of cells with micronucleated binucleated cells in the test substance-treated groups was not statistically significantly increased relative to vehicle control at any dose level ($p > 0.05$, Fisher's Exact test). The results for the positive and negative controls indicate that all criteria for a valid assay were met.

Based on the findings of this study, JA900-DAA was concluded to be negative for the induction of micronuclei in both non-activated and S9-activated test systems in the *in vitro* mammalian cell micronucleus test using human peripheral blood lymphocytes.

PURPOSE

The purpose of this study was to evaluate the potential of a test substance and/or its metabolites to induce micronuclei in HPBL using cytokinesis-block methodology in the presence and absence of an exogenous metabolic activation system. Historical control data are included in Appendix I. A copy of the study protocol is included in Appendix II.

The study was conducted in compliance with the OECD testing guideline 487 (OECD 2014).

CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The test substance, JA900-DAA, was received by BioReliance on 24 March 2015 and was assigned the code number AE19WX. Upon receipt, the test substance was described as a clear, colorless liquid and was stored at room temperature, protected from light.

The Sponsor has determined the identity, strength, purity and composition or other characteristics to define the test substance and the stability of the test substance. A copy of the Certificate of Analysis is included in Appendix III. Based on the expiration date provided in the Certificate of Analysis, the test substance is considered stable through March 2017.

The vehicle used to deliver JA900-DAA to the test system was:

Vehicle	Supplier	CAS No.	Lot No.	Exp. Date
Water	Gibco	7732-18-5	1636484	Sep 2016

Dose formulations were adjusted to compensate for the purity (51.4%) of the test substance, using a correction factor of 1.95. Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light.

Positive control components used in the study were:

Component	Supplier	CAS No.	Lot No.	Exp. Date
Cyclophosphamide (CP)	Sigma-Aldrich	6055-19-2	MKBS0021V	Oct 2017
Vinblastine (VB)	Sigma-Aldrich	143-67-9	BCBL5194V	30 June 2015
Sterile water	Gibco	7732-18-5	1636484	Sep 2016

VB was dissolved in sterile distilled water to stock concentration of 0.0005, 0.00075, and 0.001 mg/mL (final concentrations of 5, 7.5, and 10 ng/mL, respectively) as the positive control in the non-activated test system. CP was dissolved and diluted in sterile distilled water to stock concentrations of 0.25, 0.5, and 0.75 mg/mL (final concentrations of 2.5, 5, and 7.5 µg/mL, respectively) for use as the positive control substance in the S9-activated test system. Since the non-activated and S9-activated treatment groups were tested concurrently, the positive control for the non-activated 4-hour exposure was eliminated. For each positive control

substance, one dose level exhibiting a sufficient number of scorable metaphase cells was selected for analysis. The vehicle for the test substance was used as the vehicle control for each treatment group.

The vehicle and positive controls have been characterized as per the Certificates of Analysis on file with the Testing Facility. The stability of the vehicle and positive controls and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Cytochalasin B (cytoB) was dissolved in DMSO to a stock concentration of 2 mg/mL. It was used at 6 µg/mL concentration to block cytokinesis.

Dose Formulation Collection and Analysis

Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

MATERIALS AND METHODS

Test System

Peripheral blood lymphocytes were obtained from a healthy non-smoking 22-year-old adult male on 07 April 2015 for the preliminary toxicity assay and from the same donor on 21 April 2015 for the definitive assay. The donor had no recent history of radiotherapy, viral infection or the administration of drugs. This system has been demonstrated to be sensitive to the genotoxicity test for detection of micronuclei of a variety of chemicals (Clare et al., 2006).

Identification of Test System

Prior to treatment, the culture tubes were identified by the BioReliance study number, dose level, test phase, treatment condition, activation system and/or replicate design.

Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 (Lot No. 3408) was obtained from Molecular Toxicology Inc. (Boone, NC). Each bulk preparation of S9 was assayed by the supplier for sterility and its ability to metabolize at least two pro-mutagens to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 was thawed and mixed with a cofactor pool to contain 2 mM magnesium chloride, 6 mM potassium chloride, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 µL S9 per milliliter medium (RPMI 1640 serum-free medium supplemented with 100 units penicillin/mL and 100 µg streptomycin/mL and 2 mM L-glutamine).

Solubility Test

A solubility test was conducted using sterile water to determine the highest soluble or workable stock concentration up to 50 mg/mL.

Experimental Design

The *in vitro* mammalian cell micronucleus assay was conducted using standard procedures (Kirsch-Volders et al. 2000; Parry and Sors 1993; Fenech and Morley, 1986; Fenech 1993) by exposing HPBL to appropriate concentrations of the test substance as well as the concurrent positive and vehicle controls, in the presence and absence of an exogenous metabolic activation system.

Preparation of Target Cells

Peripheral blood lymphocytes were cultured in complete medium (RPMI-1640 containing 15% fetal bovine serum, 2mM L-glutamine, 100 units penicillin, 100 µg/mL streptomycin) by adding 0.5 mL heparinized blood to a centrifuge tube containing 5 mL of complete medium with 2% phytohemagglutinin. The cultures were incubated under standard conditions (37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air) for 44-48 hours.

Preliminary Toxicity Test for Selection of Dose Levels

HPBL were exposed to vehicle alone and to nine concentrations of test substance with half-log dose spacing using single cultures. Precipitation in the treatment medium was determined using unaided eye at the beginning and conclusion of treatment. The osmolality of the vehicle and that of the highest dose level in treatment medium was measured. In the absence of cytotoxicity or visible precipitate in the treatment medium, the highest selected for the definitive assay was the dose tested in the preliminary toxicity assay.

Micronucleus Assay

Four dose levels were tested using duplicate cultures at appropriate dose intervals based on the toxicity profile of the test substance. Precipitation in the treatment medium was determined using unaided eye at the beginning and conclusion of treatment. In the absence of cytotoxicity or visible precipitate in the treatment medium, the highest evaluated for micronuclei was 2000 µg/mL, which is the limit dose for this assay. Two additional dose levels were included in the evaluation.

Treatment of Target Cells (Preliminary Toxicity Test and Definitive Assay)

The pH was measured at the highest test substance concentration prior to dosing using test tape. Treatment was carried out by refeeding the cultures with 4.5 mL complete medium for the non-activated exposure or 4.5 mL S9 mix (3.5 mL serum-free culture medium + 1 mL of S9 cofactor pool) for the S9-activated exposure, to which was added 500 µL of test substance dosing solution or vehicle alone. In the definitive assay, positive control cultures were

resuspended in either 5 mL of complete medium for the non-activated studies, or 5 mL of the S9 reaction mixture (4 mL serum free medium + 1 mL of S9 cofactor pool), to which was added 50 µL of positive control in solvent.

After the 4 hour treatment in the non-activated and the S9-activated studies, the cells were centrifuged, the treatment medium was aspirated, the cells were washed with calcium and magnesium free phosphate buffered saline (CMF-PBS), re-fed with complete medium containing cytoB at 6.0 µg/mL and returned to the incubator under standard conditions. For the 24 hour treatment in the non-activated study, cytoB (6.0 µg/mL) was added at the beginning of the treatment.

Collection of Cells (Preliminary Toxicity Test and Definitive Assay)

Cells were collected after being exposed to cyto B for 24 hours (± 30 minutes), 1.5 to 2 normal cell cycles, to ensure identification and selective analysis of micronucleus frequency in cells that have completed one mitosis evidenced by binucleated cells (Fenech and Morley, 1986). The cyto B exposure time for the 4 hour treatment in the non-activated and the S9-activated studies was 20 hours (± 30 minutes).

Cells were collected by centrifugation, swollen with 0.075M KCl, washed with fixative (methanol: glacial acetic acid, 25:1 v/v), capped and were stored overnight or longer at 2-8°C. To prepare slides, the cells were collected by centrifugation and if necessary, the cells were resuspended in fresh fixative. The suspension of fixed cells was applied to glass microscope slides and air-dried. The slides were stained with acridine orange and identified by the BioReliance study number, treatment condition, dose level, test phase, harvest date, activation system, and replicate tube design.

Cell Cycle Kinetics Scoring (Preliminary Toxicity Test and Definitive Assay)

For the preliminary toxicity test, at least 500 cells were evaluated to determine the CBPI at each dose level and the control. For the micronucleus assay, at least 1000 cells (500 cells per culture) were evaluated to determine the CBPI at each dose level and the control. The CBPI was determined using the following formula:

$$\text{CBPI} = \frac{1\text{X Mononucleated cells} + 2 \text{ x Binucleated cells} + 3 \text{ x Multinucleated cells}}{\text{Total number of cells scored}}$$

$$\% \text{ Cytostasis (cytotoxicity)} = 100 - 100 \{(\text{CBPIt}-1) / (\text{CBPIc}-1)\}$$

t = test substance treatment culture
c = vehicle control culture

Micronucleus Scoring (Definitive Assay)

The slides from at least three test substance treatment groups were coded using random numbers by an individual not involved with the scoring process and scored for the presence of

micronuclei based on cytotoxicity. Whenever possible, a minimum of 2000 binucleated cells from each concentration (1000 binucleated cells from each culture) were examined and scored for the presence of micronuclei. Micronuclei in a binucleate cell (MN-BN) were recorded if they meet the following criteria:

- the micronucleus should have the same staining characteristics as the main nucleus.
- the micronuclei should be separate from the main nuclei or just touching (no cytoplasmic bridges).
- the micronuclei should be of regular shape and approximately 1/3 or less than the diameter of the main nucleus.

Statistical Analysis

Statistical analysis was performed using the Fisher's exact test ($p \leq 0.05$) for a pairwise comparison of the percentage of micronucleated cells in each treatment group with that of the vehicle control. The Cochran-Armitage trend test was used to assess dose-responsiveness.

Criteria for Determination of a Valid Test

Vehicle Controls

The frequency of cells with structural chromosomal aberrations should ideally be within the 95% control limits of the distribution of the historical negative control database. If the concurrent negative control data fall outside the 95% control limits, they may be acceptable as long as these data are not extreme outliers (indicative of experimental or human error).

Positive Controls

The percentage of micronucleated cells must be significantly greater than the concurrent vehicle control ($p \leq 0.05$). In addition, the cytotoxicity response must not exceed the upper limit for the assay (60%).

Cell Proliferation

The CBPI of the vehicle control at harvest must be ≥ 1.4.

Test Conditions

The test substance must be tested using a 4-hr treatment with and without S9, as well as a 24-hr treatment without S9. However, all three treatment conditions need not be evaluated in the case of a positive test substance response under any treatment condition.

Analyzable Concentrations

At least 2000 binucleated cells from at least three appropriate test substance concentrations.

Evaluation of Test Results

The test substance was considered to have induced a positive response if:

- at least one of the test concentrations exhibits a statistically significant increase when compared with the concurrent negative control ($p \leq 0.05$), and
- the increase is concentration-related ($p \leq 0.05$), and
- results are outside the 95% control limit of the historical negative control data.

The test substance was considered to have induced a clear negative response if none of the criteria for a positive response were met.

Electronic Data Collection Systems

Electronic systems used for the collection or analysis of data included but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel (Microsoft Corporation)	Calculations
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

Archives

All raw data, the protocol, pertinent study email correspondence, and all reports for procedures performed at BioReliance will be maintained in the archives at BioReliance, Rockville, MD for at least five years, unless otherwise requested by the Sponsor. At that time, the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials will first be copied and the copy will be retained by the BioReliance archives in accordance with the applicable SOPs. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database. The raw data, reports, and other documents generated at locations other than BioReliance will be archived by the test site. All unused test substance was disposed prior to report finalization.

Deviations

No deviations from the study protocol or assay method SOPs occurred during the conduct of this study.

RESULTS AND DISCUSSION

Solubility Test

Water was used as the vehicle based on information provided by the Sponsor, the solubility of the test substance, and compatibility with the target cells. In a solubility test conducted at BioReliance, the test substance formed a clear solution in sterile water at a concentration of approximately 50 mg/mL, the maximum concentration tested for solubility. The formulation prepared for use in the solubility test also was adjusted for test substance purity using a correction factor of 1.95.

Preliminary Toxicity Test

A preliminary toxicity test was conducted to observe the cytotoxicity profile of the test substance and to select suitable dose levels for the definitive micronucleus assay. HPBL cells were first exposed to nine concentrations of JA900-DAA ranging from 0.2 to 2000 µg/mL, as well as vehicle controls, in both the absence and presence of an Aroclor-induced S9 activation system for 4 hours, or continuously for 24 hours in the absence of S9 activation. The test substance was soluble in water and in the treatment medium at all dose levels tested at the beginning and conclusion of the treatment period. The osmolality in treatment medium was measured as follows:

Dose tested	Dose levels (µg/mL)	Osmolality (mmol/kg)
Water	0	251
Highest	2000	250

The osmolality of the test substance dose level in treatment medium is acceptable because it did not exceed the osmolality of the vehicle by more than 20%. The pH of the highest dose level of test substance in treatment medium was 7.5.

The results of the evaluation of CBPI and % cytotoxicity are presented in Tables 1, 2 and 3. Cytotoxicity [55 ± 5% cytokinesis-blocked proliferation index (CBPI) relative to the vehicle control] was not observed at any dose level in any of the treatment groups. Based on the results of the preliminary toxicity assay, the dose levels selected for testing in the micronucleus assay were as follows:

Treatment Condition	Treatment Time	Recovery Time	Dose levels (µg/mL)
Non-activated	4 hr	20 hr	250, 500, 1000, 2000
	24 hr	0 hr	250, 500, 1000, 2000
S9-activated	4 hr	20 hr	250, 500, 1000, 2000

Micronucleus Assay

In the micronucleus assay, the test substance was soluble in water and in the treatment medium at all dose levels tested at the beginning and conclusion of the treatment period. The pH of the highest concentration of test substance in treatment medium was 7.5.

Results of the cytotoxicity and micronucleus analysis in the non-activated 4-hour exposure group are presented in Table 4 and Table 7, respectively. The dose levels selected for analysis of micronucleus were 500, 1000, and 2000 µg/mL. At the highest test concentration, 2000 µg/mL, cytotoxicity was 15% relative to the vehicle control. The percentage of cells with micronuclei in the test substance-treated group was not significantly increased relative to vehicle control at any dose level ($p > 0.05$, Fisher's Exact test).

Results of the cytotoxicity and micronucleus analysis in the S9-activated 4-hour exposure group are presented in Table 5 and Table 8, respectively. The dose levels selected for analysis of micronucleus were 500, 1000, and 2000 µg/mL. At the highest test concentration, 2000 µg/mL, cytotoxicity was 17% relative to the vehicle control. The percentage of cells with micronuclei in the test substance-treated group was not significantly increased relative to vehicle control at any dose level ($p > 0.05$, Fisher's Exact test). The percentage of micronucleated cells in the CP (positive control) group (1.2%) was statistically significant ($p \leq 0.01$, Fisher's Exact test).

Results of the cytotoxicity and micronucleus analysis in the non-activated 24-hour exposure group are presented in Table 6 and Table 9, respectively. The dose levels selected for analysis of micronucleus were 500, 1000, and 2000 µg/mL. At the highest test concentration, 2000 µg/mL, cytotoxicity was 35% relative to the vehicle control. The percentage of cells with micronuclei in the test substance-treated group was not significantly increased relative to vehicle control at any dose level ($p > 0.05$, Fisher's Exact test). The percentage of micronucleated cells in the VB (positive control) group (1.1%) was statistically significant ($p \leq 0.01$, Fisher's Exact test).

The results for the positive and vehicle controls indicate that all criteria for a valid assay were met. The Common Technical Document (CTD) Summary Table is included in Appendix IV.

CONCLUSION

The positive and vehicle controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, JA900-DAA was concluded to be negative for the induction of micronuclei in the non-activated and S9-activated test systems in the *in vitro* mammalian micronucleus test using human peripheral blood lymphocytes.

REFERENCES

Clare, G., Lorenzon, G., Akhurst, L.C., Marzin, D., van Delft, J., Montero, R., Botta, A., Bertens, A., Cinelli, S., Thybaud, V. and Lorge, E. (2006), SFTG International collaborative study on the *in vitro* micronucleus test. II. Using human lymphocytes, *Mutation Res.,* 607, 37-60.

Fenech, M. and Morley, A.A. (1986). Cytokinesis-block micronucleus method in human lymphocytes: effect of *in-vivo* ageing and low dose X-irradiation. Mutation Res., 161, 193-198.

Fenech, M. (1993) The cytokinesis-block micronucleus technique: A detailed description of the method and its application to genotoxicity studies in human populations, Mutat. Res. 285:35-44.

Kirsch-Volders, M., Sofuni, T., Aardema, M., Albertini, S., Eastmond, D., Fenech, M., Ishidate, M. Jr, Lorge, E., Norppa, H., Surralles, J., von der Hude, W. and Wakata, A. (2000). Report from the *In Vitro* Micronucleus Assay Working Group. Environ. Mol. Mutagen., 35, 167-172.

OECD Guideline for the Testing of Chemicals, Guideline 487 (In Vitro Mammalian Cell Micronucleus Assay). (2014). Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, updated and adopted 26 September 2014.

Parry, J.M. and Sors, A. (1993). The detection and assessment of the aneugenic potential of environmental chemicals: the European Community aneuploidy project. Mutation Res., 287, 3-15.

TABLE 1
PRELIMINARY TOXICITY ASSAY USING JA900-DAA
IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION
4-HOUR TREATMENT, 24-HOUR HARVEST

Treatment Condition µg/mL	Total # of Cells Counted	Count per total cells Cells with # of nuclei 1	2	>2	CBPI[1]	Cytotoxicity[2]
Water	500	177	277	46	1.738	
JA900-DAA						
0.2	500	193	258	49	1.712	4%
0.6	500	174	287	39	1.730	1%
2	500	187	257	56	1.738	0%
6	500	178	274	48	1.740	0%
20	500	216	242	42	1.652	12%
60	500	170	272	58	1.776	-5%
200	500	169	293	38	1.738	0%
600	500	151	299	50	1.798	-8%
2000	500	158	300	42	1.768	-4%

[1]CBPI = Cytokinesis-Block Proliferation Index

[2]Relative to vehicle control

TABLE 2
PRELIMINARY TOXICITY ASSAY USING JA900-DAA
IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION
4-HOUR TREATMENT, 24-HOUR HARVEST

Treatment Condition µg/mL	Total # of Cells Counted	Count per total cells Cells with # of nuclei 1	2	>2	CBPI[1]	Cytotoxicity[2]
Water	500	152	315	33	1.762	
JA900-DAA						
0.2	500	161	303	36	1.750	2%
0.6	500	155	310	35	1.760	0%
2	500	228	254	18	1.580	24%
6	500	216	259	25	1.618	19%
20	500	195	286	19	1.648	15%
60	500	198	266	36	1.676	11%
200	500	211	266	23	1.624	18%
600	500	200	282	18	1.636	17%
2000	500	199	273	28	1.658	14%

[1]CBPI = Cytokinesis-Block Proliferation Index

[2]Relative to vehicle control

TABLE 3
PRELIMINARY TOXICITY ASSAY USING JA900-DAA
IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION
24-HOUR TREATMENT, 24-HOUR HARVEST

Treatment Condition µg/mL	Total # of Cells Counted	Count per total cells Cells with # of nuclei 1	2	>2	CBPI[1]	Cytotoxicity[2]
Water	500	165	241	94	1.858	
JA900-DAA						
0.2	500	156	264	80	1.848	1%
0.6	500	151	254	95	1.888	-3%
2	500	135	270	95	1.920	-7%
6	500	126	278	96	1.940	-10%
20	500	145	252	103	1.916	-7%
60	500	149	251	100	1.902	-5%
200	500	140	250	110	1.940	-10%
600	500	153	254	93	1.880	-3%
2000	500	227	250	23	1.592	31%

[1]CBPI = Cytokinesis-Block Proliferation Index

[2]Relative to vehicle control

TABLE 4

CONCURRENT CYTOTOXICITY TEST USING JA900-DAA

IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

4-HOUR TREATMENT, 24-HOUR HARVEST

Treatment Condition µg/mL	Replicate Culture	Total # of Cells Counted	Count per total cells Cells with # of nuclei			CBPI[1]	Cytotoxicity[2]
			1	2	>2		
Water	A	500	178	275	47	1.809	
	B	500	115	330	55		
JA900-DAA							
250	A	500	168	285	47	1.755	7%
	B	500	168	288	44		
500	A	500	158	296	46	1.788	3%
	B	500	150	300	50		
1000	A	500	160	293	47	1.804	1%
	B	500	140	303	57		
2000	A	500	186	284	30	1.684	15%
	B	500	184	292	24		

[1]CBPI = Cytokinesis-Block Proliferation Index

[2]Relative to vehicle control

TABLE 5
CONCURRENT CYTOTOXICITY TEST USING JA900-DAA
IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION
4-HOUR TREATMENT, 24-HOUR HARVEST

Treatment Condition µg/mL	Replicate Culture	Total # of Cells Counted	Count per total cells Cells with # of nuclei			CBPI[1]	Cytotoxicity[2]
			1	2	>2		
Water	A	500	164	306	30	1.726	
	B	500	172	296	32		
JA900-DAA							
250	A	500	158	326	16	1.665	8%
	B	502	216	264	22		
500	A	500	204	274	22	1.662	9%
	B	500	179	298	23		
1000	A	500	156	326	18	1.738	-2%
	B	500	156	312	32		
2000	A	500	227	250	23	1.600	17%
	B	500	213	270	17		
CP, 2.5	A	500	243	248	9	1.508	30%
	B	500	265	228	7		
CP, 5	A	500	307	190	3	1.328	55%
	B	500	368	132	0		
CP, 7.5	A	500	316	182	2	1.302	58%
	B	500	384	116	0		

[1]CBPI = Cytokinesis-Block Proliferation Index

[2]Relative to vehicle control

TABLE 6
CONCURRENT CYTOTOXICITY TEST USING JA900-DAA
IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION
24-HOUR TREATMENT, 24-HOUR HARVEST

Treatment Condition µg/mL	Replicate Culture	Total # of Cells Counted	Count per total cells Cells with # of nuclei 1	2	>2	CBPI[1]	Cytotoxicity[2]
Water	A	500	153	235	112	1.880	
	B	500	158	263	79		
JA900-DAA							
250	A	500	123	263	114	1.972	-10%
	B	500	118	283	99		
500	A	500	173	280	47	1.804	9%
	B	500	165	240	95		
1000	A	500	203	250	47	1.653	26%
	B	500	213	265	22		
2000	A	500	215	270	15	1.568	35%
	B	500	245	242	13		
VB, 5 ng/mL	A	500	136	254	110	1.953	-8%
	B	500	136	249	115		
VB, 7.5 ng/mL	A	500	138	248	114	1.930	-6%
	B	500	148	250	102		
VB, 10 ng/mL	A	500	407	83	10	1.227	74%
	B	500	388	100	12		

[1]CBPI = Cytokinesis-Block Proliferation Index

[2]Relative to vehicle control

TABLE 7

MICRONUCLEUS ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED WITH JA900-DAA IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

DEFINITIVE ASSAY: 4-HOUR TREATMENT, 24-HOUR HARVEST

Treatment µg/mL	Replicate Culture	Total # of Cells Counted	Percentage of Micronucleated Binucleated Cells per culture	Average Percent Micronucleated Binucleated Cells per Dose
Water	A	1000	0.1%	0.2%
	B	1000	0.2%	
JA900-DAA				
500	A	1000	0.1%	0.1%
	B	1000	0.1%	
1000	A	1000	0.2%	0.2%
	B	1000	0.2%	
2000	A	1000	0.2%	0.2%
	B	1000	0.1%	

* $p \leq 0.05$; ** $p \leq 0.01$, Fisher's exact test, relative to the solvent control.

TABLE 8

MICRONUCLEUS ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED WITH JA900-DAA IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

DEFINITIVE ASSAY: 4-HOUR TREATMENT, 24-HOUR HARVEST

Treatment µg/mL	Replicate Culture	Total # of Cells Counted	Percentage of Micronucleated Binucleated Cells per culture	Average Percent Micronucleated Binucleated Cells per Dose
Water	A	1000	0.1%	0.1%
	B	1000	0.1%	
JA900-DAA				
500	A	1000	0.2%	0.2%
	B	1000	0.1%	
1000	A	1000	0.1%	0.2%
	B	1000	0.2%	
2000	A	1000	0.1%	0.1%
	B	1000	0.1%	
CP, 5	A	1000	1.3%	1.2%**
	B	1000	1.1%	

* $p \leq 0.05$; ** $p \leq 0.01$, Fisher's exact test, relative to the solvent control.

TABLE 9

MICRONUCLEUS ANALYSIS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TREATED WITH JA900-DAA IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

DEFINITIVE ASSAY: 24-HOUR TREATMENT, 24-HOUR HARVEST

Treatment µg/mL	Replicate Culture	Total # of Cells Counted	Percentage of Micronucleated Binucleated Cells per culture	Average Percent Micronucleated Binucleated Cells per Dose
Water	A	1000	0.2%	0.2%
	B	1000	0.1%	
JA900-DAA				
500	A	1000	0.1%	0.1%
	B	1000	0.1%	
1000	A	1000	0.1%	0.1%
	B	1000	0.1%	
2000	A	1000	0.0%	0.1%
	B	1000	0.1%	
VB, 7.5 ng/mL	A	1000	1.0%	1.1%**
	B	1000	1.1%	

* $p \leq 0.05$; ** $p \leq 0.01$, Fisher's exact test, relative to the solvent control.

APPENDIX I: Historical Control Data

IN VITRO MICRONUCLEUS TEST USING
HUMAN PERIPHERAL BLOOD LYMPHOCYTES (HPBL)

HISTORICAL CONTROL VALUES
MICRONUCLEUS INDUCTION
2012-2014

NON-ACTIVATED TEST SYSTEM

Historical Values	Micronucleated Binucleated Cells (%)			
	Negative Control [1]		Positive Controls	
	4-hour	24-hour	4-hour [2]	24-hour [3]
Mean	0.37	0.37	3.75	1.80
Standard Deviation	±0.21	±0.24	±1.70	±0.91
95% Control Limits	0.00-0.80	0.00-0.86	0.35-7.15	0.00-3.62
Range [5]	0.05-1.10	0.10-2.00	1.00-10.10	0.50-5.70

S9-ACTIVATED TEST SYSTEM

Historical Values	Micronucleated Binucleated Cells (%)	
	Negative Control[1]	Positive Control [4]
Mean	0.32	1.52
Standard Deviation	±0.19	±0.45
95% Control Limits	0.00-0.69	0.63-2.42
Range [5]	0.10-1.10	0.80-3.30

1. Solvents include water, saline, DMSO, ethanol, acetone, and other non-standard and Sponsor supplied vehicles.
2. Positive control for non-activated 4 hour studies, Mitomycin C (MMC).
3. Positive control for non-activated 24 hour studies, Vinblastine (VB).
4. Positive control for S9-activated studies, Cyclophosphamide (CP).
5. Range from minimum to maximum.

APPENDIX II: Study Protocol

BioReliance by SAFC

Protocol

Study Title	***In Vitro* Mammalian Cell Micronucleus Assay in Human Peripheral Blood Lymphocytes (HPBL)**
Study Director	Shambhu Roy, PhD
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
Sponsor	International Flavors & Fragrances Inc. 800 Rose Lane Union Beach, NJ 07735
Sponsor's Authorized Representative	Xiao Huang, PhD
BioReliance Study Number	AE19WX.348.BTL

1. KEY PERSONNEL

Study Director	Shambhu Roy, PhD BioReliance Corporation Phone: 301-610-2956 Email: shambhu.roy@bioreliance.com
BioReliance Quality Assurance Representative	Karen Westray, RQAP-GLP BioReliance Corporation Phone: 301-610-2856 Email: karen.westray@bioreliance.com
Sponsor's Authorized Representative	Xiao Huang, PhD International Flavors & Fragrances Inc. 800 Rose Lane Union Beach, NJ 07735 Phone: 732-203-8136 Fax: 732-203-8176 Email: xiao.huang@iff.com

2. TEST SCHEDULE

Proposed Experimental Initiation Date	09 April 2015
Proposed Experimental Completion Date	21 May 2015
Proposed Report Date	05 June 2015

3. REGULATORY REQUIREMENTS

This study will be performed in compliance with the following Good Laboratory Practices (GLP) regulations.

- US EPA GLP Standards 40 CFR 792 (TSCA)

At a minimum, all work performed at US test site(s) will comply with the US GLP regulations stated above. Non-US sites must follow the GLP regulations governing their site. The regulations that were followed will be indicated on the compliance statement in the final contributing report.

4. QUALITY ASSURANCE

The protocol, any amendments, at least one in-lab phase, the raw data, draft report(s), and final report(s) will be audited by BioReliance Quality Assurance (QA) and a signed QA Statement will be included in the final report.

Test Site Quality Assurance (where applicable)

Test Site QA is responsible for performing an in-lab phase inspection, auditing raw data and final report(s), and providing the inspection results to the Principal Investigator, Study Director, and their respective management. A signed QA Statement documenting the type of audit performed, the dates it was performed, and the dates in which the audit results were reported to the Study Director,

Principal Investigator and their respective management must be submitted by the test site QA.

5. PURPOSE

The purpose of this study is to evaluate the potential of a test substance and/or its metabolites to induce micronuclei in HPBL using cytokinesis-block methodology in the presence and absence of an exogenous metabolic activation system. The assay design is based on the OECD Guideline 487, updated and adopted 26 September 2014.

6. TEST SUBSTANCE INFORMATION

Identification	JA900-DAA*
Synonyms	Jeffamine ED900 diacrylamide* (*The test substance is a polymer, 51% in ethanol)
Storage Conditions	Room Temperature Protect from light
Purity	51.4% (a correction factor of 1.95 will be used for dose formulations)
Average Molecular Weight (M_n)	Approximately 1000 g/mol

Characterization of Test Substance
Characterization of the Test Substance is the responsibility of the Sponsor.

Test Substance Reserve Sample
A reserve sample of the Test Substance is the responsibility of the Sponsor.

Characterization of Dose Formulations
Dose formulations will not be analyzed.

Stability of Test Substance in Vehicle
Stability of Test Substance in Vehicle, under the conditions of use, is the responsibility of the Sponsor.

Disposition of Test Substance and Dose Formulations
All unused test substance will be disposed prior to report finalization unless the test substance is used on another study. Residual dose formulations will be discarded after use.

7. TEST SYSTEM

Peripheral blood lymphocytes will be obtained from healthy adults, 18-35 years of age, non-smokers, without a recent history of radiotherapy, viral infections or the

administration of drugs. This system has been demonstrated to be sensitive to the genotoxicity test for detection of micronuclei of a variety of chemicals (Clare et al., 2006).

Peripheral blood lymphocytes will be obtained from **male** donors only.

8. EXPERIMENTAL DESIGN AND METHODOLOGY

The *in vitro* mammalian cell micronucleus assay will be conducted by exposing HPBL to appropriate concentrations of the test substance as well as the concurrent positive and vehicle controls, in the presence and absence of an exogenous metabolic activation system.

Solubility Determination

According to the sponsor, the test substance is soluble in water.

As needed, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice, selected in order of preference, will be that which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/mL for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test substance, the solvents to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

Preparation of Target Cells

Peripheral blood lymphocytes will be cultured in complete medium (RPMI-1640 containing 15% heat inactivated fetal bovine serum, 2mM L-glutamine, 100 units penicillin and 100 µg/mL streptomycin) by adding 0.5 mL heparinized blood to a centrifuge tube containing 5 mL of complete medium with 2% phytohemagglutinin. Alternate volumes of blood and media may be used if necessary. The cultures will be incubated under standard conditions (37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air) for 44-48 hours.

Identification of Test System

The cultures will be identified by the BioReliance study number and a code system to designate at least the treatment condition, dose level, and test phase.

Exogenous Metabolic Activation System

Liver Homogenate

Liver homogenate (S9) will be purchased commercially (MolTox; Boone, NC). It is prepared from male Sprague-Dawley rats that have been injected intraperitonealy with Aroclor™ 1254 (200 mg/mL in corn oil), at a dose of 500 mg/kg, 5 days before sacrifice.

S9 Mix

S9 mix will be prepared on the day of use and added to the test system at 20% (v/v). The final concentrations of the components in the test system are as indicated below.

Component	Final Concentration in Cultures
NADP (sodium salt)	1 mM
Glucose-6-phosphate	1 mM
Potassium chloride	6 mM
Magnesium chloride	2 mM
S9 homogenate	20 µL/mL

Controls

No analyses will be performed on the positive control substances or the positive control dose formulations. The neat positive control substances and the vehicles used to prepare the test substance and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

Vehicle Control

The vehicle for the test substance will be used as the vehicle control for each treatment group. For vehicles with no historical control data, an untreated control will be included.

Positive Controls

Results obtained from these substances will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test substance. One dose level of each positive control will be evaluated microscopically for micronucleus induction.

Positive Control	CAS #	S9	Time (hrs)	Concentrations*
Cyclophosphamide (CP)	6055-19-2	+	4	2.5, 5 and 7.5 µg/mL
Mitomycin C (MMC)	50-07-7	–	4**	0.4 and 0.5 µg/mL
Vinblastine (VB)	143-67-9	–	24	5, 7.5 and 10 ng/mL

*Prepared in water

** If +S9 and –S9 are tested concurrently, the positive control without S9 for the 4 hour exposure will be eliminated.

Frequency and Route of Administration

Target cells will be treated for 4 hours in the absence and presence of S9, and for 24 hours in the absence of S9, by incorporation of the test substance vehicle mixture into the treatment medium.

Preliminary Toxicity Test for Selection of Dose Levels

HPBL will be exposed to vehicle alone and to nine concentrations of test substance with half-log dose spacing using single cultures. Unless limited by solubility, the test substance will be evaluated at a maximum concentration of 2000 µg/mL or 10 mM,

whichever is lower. If limited by solubility in the vehicle, the test substance will be evaluated at the highest concentration able to be prepared and administered as a workable suspension. The osmolality of the highest dose level, lowest precipitating dose level (where applicable) and the highest soluble dose level (where applicable) in treatment medium will be measured. If the osmolality of the dose levels in the treatment medium is considered excessive (>120% of vehicle), the Sponsor will be consulted. Dose levels for the micronucleus assay will be based upon post-treatment toxicity (cytokinesis-blocked proliferation index (CBPI) relative to the vehicle control) and will be documented in the raw data and report.

Micronucleus Assay
At least four dose levels will be tested using duplicate cultures at appropriate dose intervals based on the toxicity profile of the test substance. Whenever possible, the highest dose level evaluated for the micronucleus will be selected to give 55 ± 5% cytotoxicity (CBPI relative to the vehicle control). At least two additional dose levels, demonstrating moderate to minimal or no toxicity, will be evaluated in the micronucleus assay. For poorly soluble test substance, the highest dose to be evaluated for micronucleus induction will be the concentration resulting in minimum precipitation in test medium, provided that there is no interference with scoring. The precipitation will be determined with the unaided eye at the beginning and conclusion of treatment. The maximum concentration to be evaluated in the Micronucleus assay will be the limit dose for this assay which is 2000 µg/mL or 10mM, or be expected to induce 55 ± 5% cytotoxicity (CBPI relative to the vehicle control), or be minimally insoluble (whichever is lowest).

Treatment of Target Cells (Preliminary Toxicity Test and Micronucleus Assay)
Test substance dosing solutions will be prepared immediately prior to use. The pH will be measured at the highest test substance concentration prior to dosing and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The lower concentrations may be measured and adjusted to neutral pH as needed. All test substance dosing will be at room temperature under filtered light. Treatment will be carried out by re-feeding the cultures with 5 mL complete medium for the non-activated exposure or 4 mL culture medium + 1 mL of S9 mix for the S9-activated exposure, to which will be added 50 µl of dosing solution of vehicle, test, and/or control substance. Larger volumes of dosing solution may be used as appropriate based on the compatibility to the test system. If larger volumes of dosing solutions are used, media volume will be adjusted accordingly for a total volume of 5 mL.

After the 4 hour treatment in the non-activated and the S9-activated studies, the cells will be centrifuged, the treatment medium will be removed, the cells will be washed with calcium and magnesium free phosphate buffered saline (CMF-PBS), re-fed with complete medium containing Cytochalasin B (cytoB) at 6.0 µg/mL and returned to the incubator under standard conditions. For the 24 hour treatment in the non-activated study, cyto B (6.0 µg/mL) will be added at the beginning of the treatment.

Collection of Cells (Preliminary Toxicity Test and Micronucleus Assay)
Cells will be collected after being exposed to cyto B for 24 hours (± 30 minutes), 1.5 to 2 normal cell cycles, to ensure identification and selective analysis of micronucleus frequency in cells that have completed one mitosis evidenced by binucleated cells (Fenech and Morley, 1986). The cyto B exposure time for the 4 hour treatment in the non-activated and the S9-activated studies will be 20 hours (± 30 minutes).

Cells will be swollen with 0.075M KCl and washed with fixative (methanol: glacial acetic acid, 25:1 v/v). Fixed slide may be stored overnight or longer at 2-8°C. To prepare slides, the cells will be collected by centrifugation and if necessary, the cells will be re-suspended in fresh fixative. The suspension of fixed cells will be applied to glass microscope slides and air-dried. The slides will be stained with acridine orange and identified by the BioReliance study number and a code system to designate at least the date of harvest, treatment condition, dose level, and test phase.

Cell Cycle Kinetics Scoring (Preliminary Toxicity Test and Micronucleus Assay)
For the preliminary toxicity test, at least 500 cells, if possible, will be evaluated to determine the CBPI at each dose level and the control. For the micronucleus assay, at least 1,000 cells (500 cells per culture), if possible, will be evaluated to determine the CBPI at each dose level and the control. The CBPI will be determined using the following formula:

$$CBPI = \frac{((1x \text{ Mononucleated cells}) + (2 \times \text{Binucleated cells}) + (3 \times \text{Multinucleated cells}))}{\text{Total number of cells scored}}$$

$$\% \text{ Cytostasis (cytotoxicity)} = 100 - 100 \{(CBPI_T - 1) \div (CBPI_C - 1)\}$$

T = test substance treatment culture
C= vehicle control culture

Micronucleus Scoring
The slides from at least three test substance treatment groups will be coded using random numbers by an individual not involved with the scoring process and scored for the presence of micronuclei based on cytotoxicity. Whenever possible, a minimum of 2000 binucleated cells from each concentration (if possible, 1000 binucleated cells from each culture) will be examined and scored for the presence of micronuclei.

Micronuclei in a binucleated cell (MN-BN) will be recorded if they meet the following criteria:

- the micronucleus should have the same staining characteristics as the main nucleus
- the micronuclei should be separate from the main nuclei or just touching (no cytoplasmic bridges)
- the micronuclei should be of regular shape and approximately 1/3 or less than the diameter of the main nucleus

Statistical Analysis
Statistical analysis will be performed using the Fisher's exact test ($p \leq 0.05$) for a pairwise comparison of the percentage of micronucleated cells in each treatment group with that of the vehicle control. The Cochran-Armitage trend test will be used to assess dose-responsiveness.

9. CRITERIA FOR DETERMINATION OF A VALID TEST

Vehicle Controls
The frequency of cells with structural chromosomal aberrations should ideally be within the 95% control limits of the distribution of the historical negative control database. If the concurrent negative control data fall outside the 95% control limits, they may be acceptable as long as these data are not extreme outliers (indicative of experimental or human error).

Positive Controls
The percentage of micronucleated cells must be significantly greater than the concurrent vehicle control ($p \leq 0.05$). In addition, the cytotoxicity response must not exceed the upper limit for the assay (60%).

Cell Proliferation
The CBPI of the vehicle control at harvest must be ≥ 1.4.

Test Conditions
The test substance must be tested using a 4-hr treatment with and without S9, as well as a 24-hr treatment without S9. However, all three treatment conditions need not be evaluated in the case of a positive test substance response under any treatment condition.

Analyzable Concentrations
At least 2000 binucleated cells from at least three appropriate test substance concentrations.

Maximum Concentration Evaluated
The maximum concentration evaluated for micronucleus induction must

a) produce cytotoxicity in the target range of 55 ± 5%, or
b) produce turbidity or a precipitate visible by eye at the end of the treatment with the test substance or
c) if no precipitate or limiting cytotoxicity was observed, be 10 mM, 2 mg/mL or 2 µl/mL, whichever is the lowest.

10. EVALUATION OF TEST RESULTS

A test substance will be considered to have induced a positive response if

a) at least one of the test concentrations exhibits a statistically significant increase when compared with the concurrent negative control ($p \leq 0.05$), and
b) the increase is concentration-related ($p \leq 0.05$), and

c) results are outside the 95% control limit of the historical negative control data.

A test substance will be considered to have induced a clear negative response if none of the criteria for a positive response were met.

If the response is neither clearly positive nor clearly negative, or in order to assist in establishing the biological relevance of a result, the data will be evaluated by expert judgment and/or further investigations. Any additional work will only be carried out following consultation with, and at the request of, the Sponsor.

In some cases, even after further investigations, the data set will preclude making a conclusion of positive or negative, at which time the response will be concluded to be equivocal. In such cases, the Study Director will use sound scientific judgment and report and describe all considerations.

11. ELECTRONIC DATA COLLECTION SYSTEMS

Electronic systems used for the collection or analysis of data will include but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel (Microsoft Corporation)	Calculations
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

12. REPORT

A report of the results of this study will accurately describe all methods used for generation and analysis of the data. The report will include, but not limited to information about the following:

- Test substance
- Vehicle
- Cells
- Test conditions
- Results
- Discussion of results
- Conclusion
- Appendices: Historical Control Data (negative and positive controls with means and standard deviations, 95% and 99% control limits), copy of protocol and any amendment, contributing reports (if applicable), and, if provided by the Sponsor, copies of the analyses that characterized the test substance, its stability and the stability and strength of the dosing preparations.
- Statement of Compliance
- Quality Assurance Statement
- Location of archived material
- CTD Tables (unless otherwise requested)

The report will be issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. A GLP Compliance Statement signed by the Study Director will also be included in the final report and will note any exceptions if the characterization of the test substance and/or the characterization of the dose formulations are not performed or provided. Four months after issuance of the draft report, if no communication regarding the study is received from the Sponsor or designated representative, the draft report may be issued as a final report. If all supporting documents have not been provided, the report will be written based on those that are provided.

13. RECORDS AND ARCHIVES

All raw data, the protocol, pertinent study email correspondence, and all reports for procedures performed at BioReliance will be maintained in the archives at BioReliance, Rockville, MD for at least five years, unless otherwise requested by the Sponsor. At that time, the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials will first be copied and the copy will be retained by the BioReliance archives in accordance with the applicable SOPs. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database. The raw data, reports, and other documents generated at locations other than BioReliance will be archived by the test site.

14. REFERENCES

Clare, G., Lorenzon, G., Akhurst, L.C., Marzin, D., van Delft, J., Montero, R., Botta, A., Bertens, A., Cinelli, S., Thybaud, V. and Lorge, E. (2006), SFTG International collaborative study on the in vitro micronucleus test. II. Using human lymphocytes, Mutation Res., 607, 37-60.

Fenech, M. and Morley, A.A. (1986). Cytokinesis-block micronucleus method in human lymphocytes: effect of *in-vivo* ageing and low dose X-irradiation. Mutation Res., 161, 193-198.

OECD Guideline for the Testing of Chemicals, Guideline 487 (In Vitro Mammalian Cell Micronucleus Assay). (2014). Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, updated and adopted 26 September 2014.

APPROVALS

Sponsor Approval

Xiao Huang 04/03/2015

Sponsor Representative Date

Study Director and Test Facility Management Approvals

BioReliance Study Director — Date: 07 Apr 2015

BioReliance Study Management — Date: 03-APR-2015

APPENDIX III: Certificate of Analysis

INTERNATIONAL FLAVORS & FRAGRANCES (IFF R&D) 1515 HIGHWAY 36, UNION BEACH, NJ 07735 (732) 264-4500
CREATORS AND MANUFACTURERS OF FLAVORS, FRAGRANCES AND AROMA CHEMICALS CABLE: INTERIFF NEW YORK

Certificate of Analysis

JA900-DAA; Jeffamine ED900 diacrylamide; lot RDLV28986.

JA900-DAA, lot RDLV28986, is 51.4% pure, has M_n = 1082 and meets all analytical standards set by International Flavors & Fragrances, Inc. The expiration date is 3/2017.

This material should be stored ambient, protected from light.

Lisa Veliath, PhD
Research Investigator
International Flavors & Fragrances
1515 Highway 36
Union Beach, NJ 07735
732-335-2871

March 20, 2015

APPENDIX IV: Common Technical Document (CTD) Summary Tables

2.6.7.8 Genotoxicity: In Vitro

Report Title:	*In Vitro* Mammalian Cell Micronucleus Assay in Human Peripheral Blood Lymphocytes (HPBL)				**Test Substance:**		JA900-DAA
Test for Induction of:	Micronuclei		**No. of Independent Assays:**	1	**BioReliance Study No.:**		AE19WX.348.BTL
Strains:	Human Peripheral Blood Lymphocytes (HPBL)		**No. of Replicate Cultures:**	2			
Metabolizing System:	Aroclor-induced rat liver S9		**No. of Cells Analyzed/Culture:**	1000			
Vehicles:	**For Test Substance:**	Water	**For Positive Controls:**	Water (MMC, CP, VB)	**GLP Compliance:**		Yes
Treatment:	24 hr without S9; 4 hr with 20 hr recovery period with and without S9		**Date of Treatment:**	23 April 2015 (Definitive Assay)			

Cytotoxic Effects:	In the definitive micronucleus assay, cytotoxicity [55 ± 5% cytokinesis-blocked proliferation index (CBPI) relative to the vehicle control] was not observed at any dose level in any of the three treatment groups.
Genotoxic Effects:	None.

MMC: Mitomycin C
CP: Cyclophosphamide
VB: Vinblastine

Test Substance: JA900-DAA (continued)

Metabolic Activation	Test Substance	Concentration µg/mL	CBPI[a]	Cytotoxicity[b] (% of Control)	Percentage of MNBN[c] Cells Per Total BN[d] Cells Counted
24-hr Continuous	Water	NA	1.880	NA	0.2
Treatment	JA900-DAA	500	1.804	9	0.1
Without	JA900-DAA	1000	1.653	26	0.1
Activation	JA900-DAA	2000	1.568	35	0.1
	VB	7.5 ng/mL	1.930	-6	1.1**
4-hr Treatment	Water	NA	1.809	NA	0.2
With 20 hr Recovery	JA900-DAA	500	1.788	3	0.1
Without	JA900-DAA	1000	1.804	1	0.2
Activation	JA900-DAA	2000	1.684	15	0.2
4-hr Treatment	Water	NA	1.726	NA	0.1
With 20 hr Recovery	JA900-DAA	500	1.662	9	0.2
With	JA900-DAA	1000	1.738	-2	0.2
Activation	JA900-DAA	2000	1.600	17	0.1
	CP	5	1.328	55	1.2**

MMC: Mitomycin C; CP: Cyclophosphamide; VB: Vinblastine; NA: Not Applicable; Fisher's Exact Test: * $p \leq 0.05$; ** $p \leq 0.01$.

a. CBPI = cytokinesis-blocked proliferation index
b. Relative to vehicle control
c. MNBN = micronucleated binucleated cells
d. BN = binucleated cells